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Apparent Tolerance of a Field-Collected Strain of *Myzus nicotianae* to Imidacloprid due to Strong Antifeeding Responses

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Abstract: A French strain of the tobacco aphid *Myzus nicotianae* Blackman (Homoptera: Aphididae), strain FR, showed high tolerance to imidacloprid in short-term (48-h) oral ingestion bioassays when compared to a susceptible reference strain of *Myzus persicae*, strain NS. The resulting tolerance factors were >50. Measures of the contact activity of imidacloprid by the FAO dip method failed to detect these high factors of tolerance. The tolerance factor was in general <10 when using the dip method. The resulting difference between tolerance factors could be attributed to a behavioural component to fitness between strain FR and strain NS as further experiments revealed. When measuring the effect of systemically applied imidacloprid on honeydew excretion, a 50% reduction occurred in both strain FR and strain NS at nearly the same concentration of imidacloprid, providing evidence for a similar antifeedant response in both strains. Starvation experiments revealed that the French strain was able to survive approximately 24 h longer than a reference laboratory strain of *M. persicae*. This result coincided with the fact that systemically applied imidacloprid showed the same aphicidal potential against strain FR after three days as against strain NS after two days, i.e. 24 h later. After rearing in the laboratory for six months the French strain of *M. nicotianae* lost its hardiness and also its apparent ability to tolerate imidacloprid. However, strain FR was a heterogeneous field strain and it is possible that a susceptible variant out-reproduced a more hardy variant.

These findings indicate that the type of bioassay is very important when assessing aphid populations for resistance against the chloronicotinyl insecticide imidacloprid, because of its distinct mode of action. It is obvious that an aphid dip test, i.e. FAO dip test, produces more reliable results than the different kinds of short-term oral ingestion bioassays, because of the reversible behavioural changes induced by imidacloprid after oral uptake. Thus a short-term oral ingestion bioassay (≤ 48 h) is not recommended for precise detection of possible resistance of *Myzus* sp. to imidacloprid, although this mode of uptake for imidacloprid might be sometimes more realistic in terms of field behaviour. The ideal test to generate most accurate data would be a slightly longer (72-h) feeding bioassay, perhaps used in conjunction with a dip test. The possible influence of the results on resistance monitoring is discussed.

Key words: imidacloprid, behaviour, *Myzus* sp., bioassay, hardiness, starvation

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1 INTRODUCTION

One of the major problems in modern agriculture is the growing tendency of insect pests to become more and more resistant to a wide variety of insecticidal classes. The peach-potato aphid, *Myzus persicae* Sulzer, and the tobacco aphid, *Myzus nicotianae* Blackman, are two closely related homopteran pests and resistance to conventional insecticides is now widespread in both species.[1,2] They showed the same mechanisms of resistance against organophosphates, carbamates and pyrethroids, i.e. elevated levels of carboxylesterase E4 or FE4, depending on karyotype.[3] The genes of esterase E4 and FE4 in the two species are identical.[4] Additionally a pirimicarb- and triazamate-insensitive acetylcholinesterase has very recently been reported as a new resistance mechanism in strains of *M. persicae* and *M. nicotianae* from different parts of the world.[5,6]

The new chloronicotinyl insecticide, imidacloprid, is now registered in 60 countries and fulfils the ambition of an agrochemical company in developing compounds with a new or unconventional mode of action to combat highly resistant insect pests.[7] Like nicotine, the compound acts as an agonist of the nicotinic acetylcholine receptor.[8] In contrast to nicotine, however, it is highly specific to insect receptors, as studies on vertebrate receptors have revealed.[9] The physicochemical properties of imidacloprid and the corresponding biological profile as well as some peculiarities, e.g. the antifeedant effect on aphids and the ability to decrease the fertility of aphids at extremely low concentrations, are well documented.[10–13] Imidacloprid lacks any bonds susceptible to attack by the common esterase-based mechanism of resistance in aphids and therefore it is a valuable tool in resistance management strategies.[14]

The tobacco-feeding form of *M. persicae*, *M. nicotianae*, was first described using multivariate morphometrics by Blackman in 1987.[15] Although originally described as anholocyclic it is now well established that a holocycle is possible in certain countries, and that *M. nicotianae* and *M. persicae* can interbreed and produce fertile eggs.[16] *M. nicotianae* has been described as a serious pest in several crops, in particular on tobacco plants, and most strains examined have been more or less resistant to conventional insecticides.[1,17] In 1988 estimated losses of 10–15 million dollars in the North Carolina tobacco crop were attributed to this single pest.[17]

One study of a red-coloured morph of a highly resistant tobacco-feeding form of *M. persicae* from Japan, closely related to *M. nicotianae*, revealed only low ratios of tolerance against imidacloprid, nicotine and cartap in oral ingestion as well as in dip bioassays.[6] The results of a monitoring programme running for several years, using discriminating concentrations of imidacloprid in contact bioassays, revealed only minor differences between populations of *M. persicae* and *M. nicotianae* from all over the world.[14] Other investigations demonstrated that *M. nicotianae* from around the world showed low levels of resistance to imidacloprid and nicotine, but that some clones seemed to be less susceptible to the recently described antifeedant effect of imidacloprid.[12,13] *M. nicotianae* feeds on a plant containing considerable amounts of nicotine and one might speculate that, in the course of evolution, this could have an impact on nicotinic acetylcholine receptor structure, although nicotine is transported in the xylem while *Myzus* sp. prefers the phloem as a feeding site.[18] There is a single publication which reports measurements of the affinity of imidacloprid to its receptor in susceptible and resistant (tobacco-associated) *M. persicae* from Japan, but no differences could be detected.[6]

In November 1994 we received a strain of *M. nicotianae* collected from a tobacco field in France with apparent tolerance to imidacloprid when compared to a susceptible reference strain of *M. persicae*. The tolerance factor for imidacloprid in this French strain of *M. nicotianae* depends on the type of bioassay (application method) used. The present study was performed to investigate possible causes of these differing tolerance factors among contact bioassays (FAO dip), systemic tests and an artificial double membrane feeding bioassay.

2 MATERIALS AND METHODS

2.1 Insecticides

All insecticides used were technical grade. Nicotine was purchased from Sigma (St. Louis, USA). Pirimicarb was obtained from Promochem GmbH (Wesel, Germany). All other compounds were from Bayer AG (Leverkusen, Germany), except pymetrozine which was from Ciba Geigy (Basel, Switzerland). Insecticidal stock solutions were prepared in acetone or water, depending on the bioassay, and subsequently diluted with an aqueous solution of 'Triton' X-100 (1 g litre^{-1}), when using the FAO dip test, pure water for the systemic test or sucrose solution (150 g litre^{-1}) for the artificial double membrane feeding bioassay.

2.2 Aphids

The tobacco aphid, *Myzus nicotianae* (strain FR), was collected from a tobacco field in the Rhone valley in France (provided by Yves Bouchery, INRA, Colmar, France). The susceptible strain (NS) of the peach-potato aphid, *Myzus persicae*, has been reared in the laboratory since 1967 under the conditions described below. Both strains were reared on Chinese cabbage at 22–23°C, 60% RH and a 16 : 8 h light : dark photoperiod.

2.3 Bioassays

All the bioassays described below were done in the laboratory under controlled conditions, i.e. 21–22°C, 45–55% RH and an ambient photoperiod. All insecticidal tests were repeated at least three times with two or three replicates of five to six concentrations (30–45 aphids) in each bioassay. Aphids not able to move in a coordinated manner (irreversible symptoms) were scored as dead. The starvation experiment was performed three times with 45 aphids in each bioassay. Lethal concentration values were calculated from probit regressions using the POLO computer program (LeOra Software, Berkeley, USA).

2.3.1 FAO dip test

The contact activity of the insecticides used was tested using a modified version of the FAO dip test.[19] Apterous adults of *Myzus* sp. were dipped for 5 s in insecticidal solutions containing 'Triton' X-100 (0·2 g litre^{-1}). After dipping, the aphids were transferred to freshly excised cabbage leaves, the petioles of which were immersed in a small tube containing pure water. Percentage mortality was scored 24 and 48 h post-dip.

2.3.2 Sachet test

The activity of the insecticidal compounds after oral ingestion was tested with a modification of an artificial double-membrane feeding bioassay, called the sachet test.[20,21] The insecticides were diluted in an aqueous solution of sucrose (150 g litre^{-1}). The prepared solution (0·4 ml) was pipetted between two layers of stretched Parafilm® which formed the sachet. Groups of 10 to 15 aphids, which had been starved for 4 h prior to the bioassay, were placed into small Petri dishes (diameter 2·8 cm). These were sealed by stretching the prepared sachets across the top. A piece of yellow cellophane was placed over the sachet to enhance the feeding activity of the aphids. Percentage mortality was scored after 24 and 48 h.

2.3.3 Systemic test procedure

The systemic test procedure used was recently described.[12] The cut petioles of excised cabbage leaves were immersed in small tubes of aqueous dilutions of imidacloprid. After a 4-h equilibration phase, each leaf was infested with 15 aphids. A filter-paper disc was placed under the leaves to catch the honeydew excreted over each 24-h period. Mortality was assessed after 24, 48 and 72 h. After each assessment the filter paper disc was replaced and stained with a 1 g litre^{-1} solution of ninhydrin in acetone to make the excreted honeydew droplets visible as dark purple spots.

2.3.4 Starvation test

The hardiness of adults of the different strains used was determined in starvation experiments. Aphids of similar size and nutritional status of each strain were placed in groups of 15 individuals in Petri dishes without a food source and the percentage mortality was scored at different time intervals.

3 RESULTS AND DISCUSSION

3.1 Sachet test

The insecticidal activity of imidacloprid by oral ingestion on strain FR was apparently rather poor in comparison to the very good effect against the susceptible *M. persicae* strain NS (Table 1). A similar result was obtained with nicotine, another agonist of the nicotinic acetylcholine receptor. The slopes of the dose response curves were less steep for strain FR than for strain NS, especially in the case of imidacloprid, which suggests a considerable heterogeneity in the field-collected population of strain FR. For this strain, tolerance factors for the two organophosphates tested, methamidophos and oxydemeton-methyl, were low, thus indicating only a low level of tolerance to organophosphates in strain FR. Apart from the insecticides mentioned above, we also

TABLE 1
Activity of Different Insecticides on *Myzus persicae* (NS) and *Myzus nicotianae* (FR) using an Artificial Double Membrane Feeding Bioassay (Sachet Test)

	Strain NS			*Strain FR*			
Insecticide	LC_{50} (*48 h*) (*mg litre*$^{-1}$)	*95% CL*[a]	*Slope*	LC_{50} (*48 h*) (*mg litre*$^{-1}$)	*95% CL*	*Slope*	*TF*[b]
Imidacloprid	0·073	0·046–0·11	1·24	14	3·9–108	0·495	192
Nicotine	4·5	1·7–8·5	1·21	>100	—	—	>22
Methamidophos	1·5	0·73–3·2	1·71	3·4	0·91–12	1·08	2
Oxydemeton-methyl	0·41	0·31–0·53	1·71	3·2	0·73–16	1·30	8
Pymetrozine	1·6	1·2–2·0	2·13	>100	—	—	>63

[a] 95% confidence limits.
[b] Tolerance factor, LC_{50} for strain FR/LC_{50} for strain NS.

tested pymetrozine, a new aphicide which acts by interruption of the aphid feeding process.[22] Using pymetrozine we also found a high tolerance in strain FR relative to strain NS when scoring for mortality in the oral ingestion bioassay after 48 h.

3.2 FAO dip test

In contrast to the results of the oral ingestion bioassay, the FAO dip test revealed only a tolerance factor of 9 to imidacloprid by strain FR, indicating that there was indeed a degree of tolerance in this tobacco-feeding strain. As with the feeding bioassay, the slope of the dose response curve of the FAO dip test was lower for strain FR than for strain NS. The results in Table 1 and Table 2 revealed that the tolerance factor between aphid strains against imidacloprid depends strongly on the bioassay procedure used.

3.3 Systemic test

Bioassays with aphids placed on cut cabbage leaves with their petioles immersed in imidacloprid solutions revealed a high degree of tolerance of the French strain FR towards imidacloprid (Fig. 1). This result agreed with the findings of the sachet test. After 48 h the LC_{50} value of imidacloprid for strain FR was >10 mg litre^{-1} whereas strain NS showed an LC_{50} value of 0·21 mg litre^{-1}, giving a tolerance factor of >47. The results from either oral ingestion bioassay, i.e. sachet test and systemic test, suggest that the French strain of *M. nicotianae*, FR, is highly tolerant to imidacloprid in different types of short-term ($\leq$48 h) oral ingestion bioassays. We know from our previous work that imidacloprid also acts as an antifeedant on aphids after oral administration of small amounts of active ingredient.[12] Thus we measured the effect of different concentrations of systemically applied imidacloprid on the honeydew excretion activity of strain FR relative to the excretion activity in our susceptible reference strain NS. The result of these tests clearly show that imidacloprid has a strong effect on the feeding behaviour of both aphid strains (Table 3). However, there is no significant difference in the calculated EC_{50} (48 h) values for the reduction in honeydew excretion between strain FR and strain NS, indicating that within the 48-h test period, imidacloprid has the same potential as an antifeedant for both strains. As displayed in Fig. 1 the French strain showed a similar dose mortality curve for imidacloprid three days after infestation as the susceptible strain had after two days. However, the real end-point in this bioassay could not be estimated for both strains, because the leaves showed first signs of senescence after four days. Abbot-corrected mortality data for strain NS revealed that LC_{50} values of both strains came closer together, i.e. the tolerance factor dropped considerably, to values between 15 and 20 after three days. Further experiments would be needed to determine the time-course of behavioural effects and mortality over a longer period of time using other field-collected strains of *M. nicotianae*. The French strain showed the same degree of mortality in the concentration range from 0·1 to 10 mg litre^{-1} after 48 h and most of the individuals

TABLE 2
Activity of Imidacloprid in Aphid Dip Bioassays (FAO Dip Test)

Species	LC_{50} *(48 h)* (*mg litre*$^{-1}$)	*95% CL*[a]	*Slope*	*TF*[b]
M. persicae (NS)	1·7	1·3–2·3	2·10	—
M. nicotianae (FR)	16	4·8–46	1·21	9

[a] 95% confidence limits.
[b] Tolerance factor, LC_{50} for strain FR/LC_{50} for strain NS.

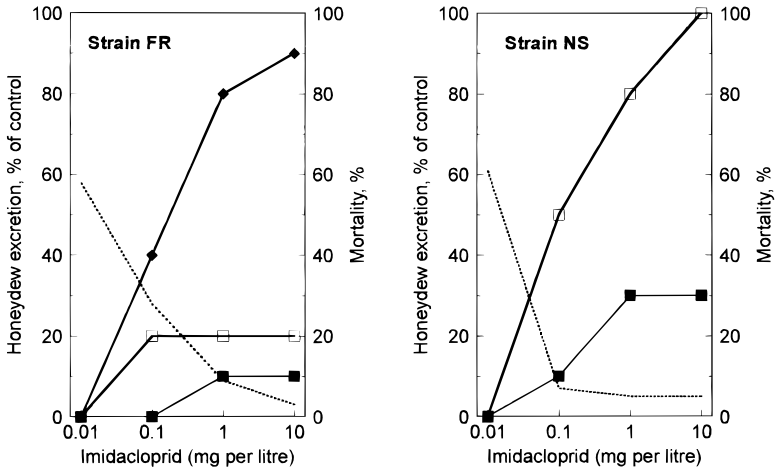

Fig. 1. Dose–response relationship for imidacloprid against *Myzus persicae* (strain NS) and *Myzus nicotianae* (strain FR) after systemic application through cut cabbage leaf petioles. Aphids dead or with irreversible symptoms were scored after (■) one day, (□) two days and (◆) three days. (– – –) Honeydew excretion was evaluated after one day.

TABLE 3
Reduction in Honeydew Excretion of *Myzus persicae* (NS) and *Myzus nicotianae* (FR) 24 h after Systemic Application of Imidacloprid through Cut Cabbage Leaf Petioles (Systemic Test)

Species	EC_{50} (48 h)[a] (*mg litre*$^{-1}$)	*95% CL*[b]	*Slope*	*TF*[c]
M. persicae (NS)	0·0157	0·002 94–0·0676	1·25	—
M. nicotianae (FR)	0·0272	0·008 37–0·0760	0·875	1·7

[a] Effective concentration, where honeydew excretion is reduced by 50% from control.
[b] 95% confidence limits.
[c] Tolerance factor, LC_{50} for strain FR/LC_{50} for strain NS.

were not feeding and were restless. After 72 h, the dose-response curve rises, suggesting that some of the aphids, which showed restless behaviour at the different concentrations, starved to death. The time after which 50% of the aphids of strain FR on cabbage leaves treated systemically with 1 mg litre^{-1} imidacloprid died was 52–56 h.

3.4 Starvation test

In the next experiment we studied the relative hardiness of the French strain compared to strain NS in order to clarify the delayed response of strain FR in systemic bioassays. We performed some simple experiments where the aphids were placed in a Petri dish without access to a food source, to determine how long the French strain is able to survive without food or water. The French *M. nicotianae* strain FR survived starvation better than the reference laboratory strain NS (Table 4), thus explaining to some extent the high tolerance factors of strain FR in short-term oral ingestion bioassays. Note that the LT_{50} value of 56·9 h (Table 4) for strain FR in starvation experiments reflects the LT_{50} value determined for the same strain after systemic application of 1 mg litre^{-1} imidacloprid through cut cabbage leaf petioles. Another starvation experiment was performed five months later and revealed that strain FR lost its hardiness when reared on Chinese cabbage in the laboratory. At present it can only be speculated that this loss is associated with suboptimal rearing conditions, i.e. strain FR was maintained on Chinese cabbage and not on tobacco, the original host plant in the field. The loss of starvation hardiness could be due to a phenotypic change, but it is also possible that strain FR was merely a heterogeneous population in which a susceptible variant out-reproduced a hardy, tolerant variant. Further experiments with other hardy strains of *M. nicotianae* should clarify which of the above-mentioned reasons is responsible for the observed loss of starvation hardiness.

TABLE 4
Survival Times of Different *Myzus* sp. Strains without Access to a Food Source (Starvation Bioassay)

Species	*Strain/clone*	LT_{50} (h)[b]	*95% CL*[c]
M. persicae	NS	37·4	35·8–39·0
M. nicotianae	FR (2 months)[a]	56·9	55·1–58·9
M. nicotianae	FR (7 months)[a]	38·2	35·4–41·0

[a] Duration of rearing of strain FR in our laboratory.
[b] Time in hours after which 50% of the aphids died.
[c] 95% confidence limits.

4 CONCLUSIONS

The present investigation revealed that with short-term bioassays ($\leq$48 h), the only reliable measure of imidacloprid tolerance is that made using an aphid dip technique, e.g. the FAO dip test which has been used in the past.[14] At present it remains unclear whether the low factor of tolerance of strain FR to imidacloprid in the applied dip test is a real mechanism-based tolerance or natural variation between closely related species. The results obtained with the organophosphate methamidophos indicated that our laboratory strain of *M. persicae* (reared for approx. 30 years in the laboratory) is not over-susceptible and is therefore a suitable reference. The tolerance factor of imidacloprid determined in a French field strain of *M. nicotianae* in the dip bioassay is comparable to those published very recently for some other strains of *M. nicotianae* and tobacco-associated forms of *M. persicae* from other parts of the world.[5,13,14] Short term bioassays, e.g. 24 h or 48 h, where oral ingestion of the active ingredient is necessary in order to detect possible tolerance, cannot be recommended without prior testing of the hardiness of the aphids, because of imidacloprid's ability to act as an antifeedant.[12] The tolerance factors in such bioassays could depend more on behavioural characteristics of the considered aphid strains than on the actual toxicity of imidacloprid. If oral ingestion bioassays for tolerance detection are unavoidable, scoring mortality after 72 h

or even better after 96 h would be recommended. In contrast to an aphid dip test, the oral ingestion bioassay mainly measures the reversible behavioural alterations in aphids which cause them to die due to starvation. If tolerance is measured in such kinds of bioassay, then it could be interpreted as behavioural tolerance, induced by the avoidance of imidacloprid-treated plants or by starvation tolerance (hardiness) of the aphids as shown in our investigation. Thus it is safer to choose the aphid dip technique for determining possible tolerance, because it focuses more on the fast-acting neurotoxicological mode of action which causes death with clearly visible symptoms resulting from interference with the nervous system. Apart from the lack of possible information on aphid behaviour, the FAO dip test is quick, reliable and produces repeatable results as demonstrated in other studies.[14] In order to build up a more distinct picture of tolerance mechanism, it could be useful sometimes to run simple measures of contact efficacy alongside assays which allow the aphid some 'choice' in its response. However, because of the fact that we looked at only two strains of *Myzus* sp. it is not possible to declare the starvation hardiness as a common mechanism of tolerance to the effects of chloronicotinyls; it is much more an example of how the expression of behaviour may decrease the efficacy of insecticides and that such behaviour could be lost after a few months of rearing in the laboratory.

The findings outlined in the present report should also be considered when assessing whitefly populations or other sucking pests for possible resistance against imidacloprid. Differences in the hardiness of different populations could strongly affect the results of resistance monitoring, if imidacloprid has the same distinct mode of action on other homopteran pests as on aphids, i.e. neurointoxication with clearly visible symptoms after a few hours at recommended field dose rates and the more inconspicuous antifeeding response in sublethal concentrations, causing death by starvation over a longer period of time.

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